RESEARCH ARTICLE

Composition dynamics of epilithic intertidal bacterial communities exposed to high copper levels

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Abstract

Copper has a dual role for organisms, both as micronutrient and toxic element. Copper mining activities have an enormous ecological impact because of the extraction process and the consequent release of copper-containing waste materials to the environment. In northern Chile, mainly in the Chañaral coastal area, this phenomenon is clearly evident. The released waste material has caused a strong modification of the area, and copper enrichment of beaches and rocky shores has provoked a decrease in the richness and diversity of many species of macroorganisms. However, the effects that copper enrichment has on microbial (e.g. bacterial epilithic) communities associated with the rocky shore environment are poorly understood. Using a culture-independent molecular approach, field sampling and laboratory microcosm experiments, we determined the effects of copper enrichment on bacterial communities inhabiting the rocky shore environment. Field samples showed a strong effect of copper on the structure of the natural bacterial epilithic communities, and microcosm experiments demonstrated rapid changes in bacterial community when copper is added, and reversibility of this effect within 48 h after copper is removed.

Introduction

Copper is an essential micronutrient for all living organisms, required at trace levels for the correct functioning of the cellular metabolism (García-Horsman *et al.*, 1994; Solioz & Stoyanov, 2003). High concentrations, however, are toxic to cells because copper may replace other metals in the metal-binding sites, interfering with the correct functioning of proteins (Kershaw *et al.*, 2005), and may trigger an overproduction of reactive oxygen species that lead to nucleic acid cleavage, lipid peroxidation and protein oxidation (Rosen, 2002). The homeostasis of copper is tightly regulated, and any imbalance decreases organism fitness.

Copper concentrations in seawater vary widely (Haraldsson & Westerlund, 1988; Bryan & Langston, 1992; Correa *et al.*, 1996), but copper concentrations between 0.5 and 3 μg L^{-1} for pristine coastal seawaters are commonly found (Lewis, 1995). Although toxic levels of copper are produced by anthropogenic activities such as the use of copper-containing algaecides, fertilizers, organic manures, and fungicides (Yruela, 2005; Bona *et al.*, 2007), one of the major external inputs of copper to marine coastal systems is mining. In numerous places around the world, copper-containing mine wastes have been reported as the cause of negative effects (Bryan & Langston, 1992; Castilla, 1995; Correa *et al.*, 1999; Marsden & DeWreede, 2000; Grout & Levings, 2001).

Microorganisms have developed different mechanisms to deal with copper (metal) excess (reviewed by Silver & Phung, 1996; Bruins *et al.*, 2000 and Nies, 2003). The mechanisms include sequestering of metals by metallothioneins (Camakaris *et al.*, 1999; Blindauer *et al.*, 2002), trafficking of metal ions by metallochaperones (Harrison *et al.*, 2000; Rosenzweig, 2002), and the efflux of metals by P-type cation-transporting ATPases (Lutsenko & Kaplan,

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1995). Efflux systems represent the largest category of resistance systems and are often highly specific for the metal(s) they export (Nies & Silver, 1995; Argüello *et al.*, 2007). Copper P-type ATPases are encoded by the *cop*A gene, and orthologs of this gene have been detected in many organisms, such as *Homo sapiens* (Bull *et al.*, 1993; Vulpe *et al.*, 1993), *Saccharomyces cerevisiae* (Fu *et al.*, 1995), *Enterococcus hirae* (Solioz & Odermatt, 1995), *Synechococcus* sp. (Kanamaru *et al.*, 1994), *Helicobacter pylori* (Ge *et al.*, 1995) and *Escherichia coli* (Petersen & Møller, 2000; Rensing *et al.*, 2000).

A well-known example of copper enrichment of coastal marine ecosystems affects Chañaral bay in northern Chile (26°15′ S; 69°34′ W) (Fig. 1). The discharge of untreated copper mining wastes in the bay began in 1939 and ended in 1990 (Correa *et al.*, 1999), causing a dramatic decrease in algal and invertebrate species richness and diversity on the intertidal zones around the impacted area (Medina *et al.*, 2005). Currently, there is still a discharge of treated copper mining wastes (denominated 'clear waters') that was moved from the initial discharge point (near to Achurra, see Fig. 1) to a new discharge point (near to Palito, see Fig. 1), which does not include particulate material but still has high dissolved copper levels (Andrade *et al.*, 2006). Concentrations of dissolved copper in the coastal waters of the Chañaral area are around 9 μg Cu L^{-1} (Correa *et al.*, 1999). As copper interactions with the biota are determined by the so-called bioavailable fraction (the fraction of copper that is not bound to any ligand, and therefore available for the biota), it is worth mentioning that bioavailable copper in the Chañaral area is around 4.4 μg Cu L^{-1}, ten times higher than the values (0.5 μg Cu L^{-1}) measured in reference waters (Andrade *et al.*, 2006). Thus, the Chañaral area represents a unique case as a valuable natural laboratory to study the effects of copper mine wastes on the coastal biota (Lee *et al.*, 2002; Stauber *et al.*, 2005). In fact, studies on the ecological effects of the long-term copper exposure in the area have indicated that copper is, by far, the main factor responsible for the decrease in biological richness and diversity of macroorganisms, specially macroalgae and invertebrates (Correa *et al.*, 1999; Medina *et al.*, 2005). Similarly, studies have also shown that copper enrichment in this area affects richness and diversity of bacterial communities, but the effects appear to be milder as compared to those reported in macroorganisms (Morán *et al.*, 2008; Hengst *et al.*, 2010). There is evidence of the same trends in other environments affected by copper pollution (Massieux *et al.*, 2004; Boivin *et al.*, 2005; Pavissich *et al.*, 2010). However, the effect that copper has on bacterial epilithic communities in intertidal zones (the bacterial communities that inhabit the rock surface and thus are directly exposed to seawater) is poorly understood. Such communities represent an interesting ecosystem because they provide not just physical and nutritional support to the higher trophic levels in

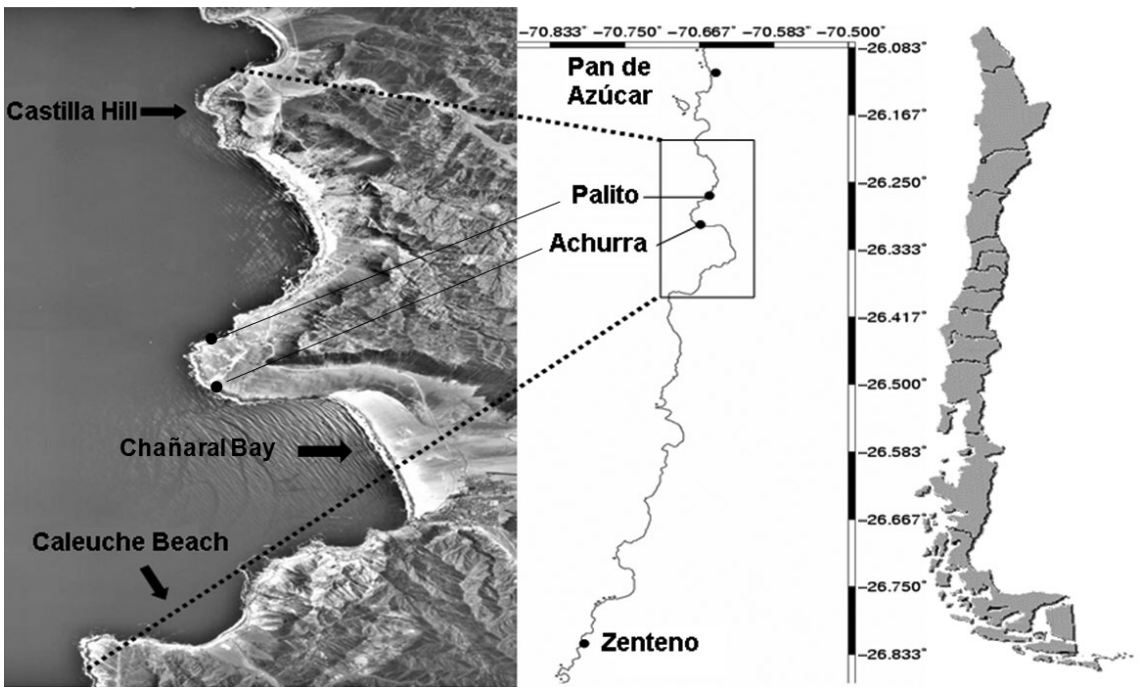

Fig. 1. Geographic location of the Chañaral area and the sampling sites.

marine ecosystems (Gorbushina, 2007), but can also participate/influence algae and invertebrate larvae settlement and development (Egan *et al.*, 2001; Matsuo *et al.*, 2003; Wahl, 2008). Therefore, a research effort on the effects of copper pollution on epilithic bacterial communities is required not only to gain an insight into the changes in these communities but also to provide clues on potential secondary effects on the macroorganisms inhabiting the area.

Here, we report the effect of exposure to high levels of copper on epilithic bacterial community composition using a culture-independent approach involving taxonomic (16S rRNA gene) and functional (*cop*A gene for copper resistance) molecular markers in field samples and microcosm experiments.

Materials and methods

Sampled localities and sampling procedures

Four localities were selected for sampling (Fig. 1). Palito (26°15.8′S; 70°40.6′W), closer to the current discharge point, and Achurra (26°18.4′S; 70°39.8′W), closer to the old discharge site, were selected as impacted sites. Zenteno (26°54.1′S; 70°48.5′W) and Pan de Azúcar (26°08.2′S; 70° 39.3′W), 45 km south and 15 km north from Chañaral bay, respectively, were selected as nonimpacted sites. Hereafter, Palito and Achurra will be referred to as sites I-1 and I-2, and Zenteno and Pan de Azúcar as sites NI-1 and NI-2, respectively. In each site, five 20 cm^2 of exposed rock surface samples were collected at random positions during low tide using a hammer and a chisel. Samples were collected in four different campaigns during January and August 2005 and January and August 2006. Each sample was put in 50-mL sterile polypropylene plastic tubes and stored at 4 °C for transporting. For the microcosm experiments, fragments of rocks were collected as described and stored at 4 ° C in sterile plastic bags for transporting.

DNA extraction

Rock samples were sonicated in 20 mL 1× TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8) in an ultrasound bath (Tru-Sweep™; Crest, NJ) for 10 min in the same plastic tubes used for transport. The liquid was then collected in sterile 50-mL polycarbonate centrifuge tubes (Nalgene Company, Rochester, NY), centrifuged for 15 min at 18 000 ***g***, and the pellet re-suspended in 200 μL of 1× TBE buffer. A modified version of the method for DNA extraction described by Ausubel *et al.* (1994) was used for rock samples, as described in Morán *et al.* (2008). DNA yields ranged from 0.125 to 1 μg cm^{-2} of exposed rock surface.

Terminal restriction fragment length polymorphisms (T-RFLP) analyses

For 16S rRNA genes analysis, primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Lane, 1991) and 1392R (5′-ACG GGC GGT GTG TAC-3′) (Lane *et al.*, 1985) were used as previously described (Morán *et al.*, 2008). Primer 8F was labeled at the 5′ end with the 2′-chloro-5′-fluoro-7′,8′-fused phenyl-1,4-dichloro-6-carboxyfluorescein fluorochrome (NED). 16S rRNA gene PCR products were digested by overnight incubation at 37 °C with 20 U of the MspI or HhaI endonucleases, in a final volume of 20 μL. Each PCR product was digested separately with each enzyme to assess for T-RFLP profiles consistency. As both profiles indicate the same trends, only the MspI profiles are reported. For functional profiling, primers copAUF (5′-GGT GCT GAT CAT CGC CTG-3′) and copAUR (5′-GGG CGT CGT TGA TAC CGT-3′) were used for the amplification of copper P-Type ATPase as previously described (De la Iglesia *et al.*, 2010). Primer copAUF was labeled at the 5′ end with the 6-carboxyfluorescein (FAM) fluorochrome. copA PCR products were digested by overnight incubation at 37 °C with 20 U of the AluI endonuclease, in a final volume of 20 μL. T-RFLP data were handled and statistically analyzed as previously described (Morán *et al.*, 2008).

Microcosm experiments

Two sets of microcosm experiments were carried out. Microcosms were mounted in acid-washed polypropylene bottles (1 L) containing about 200 cm^2 of exposed rock surface and 500 mL of 0.22-μm-filtered seawater. The liquid portion, with or without copper, was replaced every 48 h to keep the initial copper concentrations of each treatment, thus avoiding metal sequestration by the rock minerals (data not shown). Before starting each experiment, all microcosms were acclimated for 24 h using 0.22-μm-filtered seawater, under the same conditions defined for the experiments. Samples were incubated at 16 °C under agitation at 100 r.p.m., with a 12/12 h light: dark regime. All treatments were performed in triplicate, and copper was added using a stock solution of $CuCl_2$ at 1 000 000 μg L^{-1}. In the first set of experiments (hereafter named *Response Experiment*), samples from sites I1 and NI-2 were incubated for 48 h with 50 or 100 μg Cu L^{-1}. The copper concentrations were chosen based on the maximum copper concentrations currently detected in the area (50 μg Cu L^{-1}) and on the maximum copper concentration historically detected in the area (100 μg Cu L^{-1}). For the second set of experiments (hereinafter *Reversion Experiment*), samples from site NI-2 were first incubated for 48 h with 50 or 100 μg Cu L^{-1}.

After that initial incubation, one set of samples was incubated for an additional 48 h at the same copper concentration, and the other set was incubated for an additional 48 h in medium with no added copper. In the case of the *Response Experiment*, samples were taken after 48 h of incubation, whereas for the *Reversion Experiment*, samples were taken at 48 and 96 h of incubation. Samples were then subjected to DNA extraction using the protocol described earlier. 16S rRNA gene and *copA* gene T-RFLP profiles were generated for each sampling time.

Results and discussion

Long-term copper exposure produces a difference in the composition of the intertidal epilithic bacterial communities

To understand whether long-term copper exposure affects the composition of intertidal epilithic bacterial communities, 16S rRNA gene T-RFLP profiles were generated for each site and sampling time. As the profiles obtained with the two restriction enzymes used generate the same trends, only the MspI profiles are discussed. The profiles from impacted and nonimpacted samples were compared. Even when the resulting profiles showed clear qualitative differences between sites (see Supporting Information, Fig. S1), no significant differences for richness (R), diversity (H), and evenness (E) values were detected (ANOVA, $P > 0.05$) between impacted and nonimpacted samples along the study time (data not shown). This observation was in agreement with previous studies in the same area but for different compartments (i.e. seawater, sediments, and algal surfaces) where no significant differences on R, H, and E values were also observed for bacterial communities, using the same approaches (Morán *et al.*, 2008; Hengst *et al.*, 2010). Thus, it seems clear that classical ecological indexes show poor discriminatory power to differentiate microbial communities in contrast with their utility to discriminate even subtle changes in macroorganism communities. This leads to the use of combined parametric and nonparametric analyses as a more robust way of analyzing changes in microbial community composition (Bohannan & Hughes, 2003). In agreement with this, cluster analysis of the T-RFLP profiles showed a clear grouping of samples according to copper exposure, for all sampling times analyzed (Fig. 2). The statistical validation of the clustering was carried out by an analysis of similarity (ANOSIM). ANOSIM comparisons between impacted and nonimpacted groups indicated that the observed grouping was statistically supported, with R values of 0.485 for 5 January, 0.413 for 5 August, 0.406 for 6 January, and 0.452 for 6 August. All the R values were significant ($P < 0.05$). The fact that all R values were around 0.4 indicates that the observed differences in taxonomic composition were moderated but consistent after long-term copper exposure, as they were observed in all four different sampling times. Whether or not the differences persist in time should be revealed using longer time series. To determine whether the observed effect in the taxonomic composition of the bacterial communities was also observed for functional composition, *cop*A gene-based T-RFLP profiles for the same samples were analyzed. In this case, significant differences in H values were detected after ANOVA analysis ($P < 0.05$) between impacted and nonimpacted groups, but not for R or E values (data not shown). Cluster analysis showed a clear grouping of

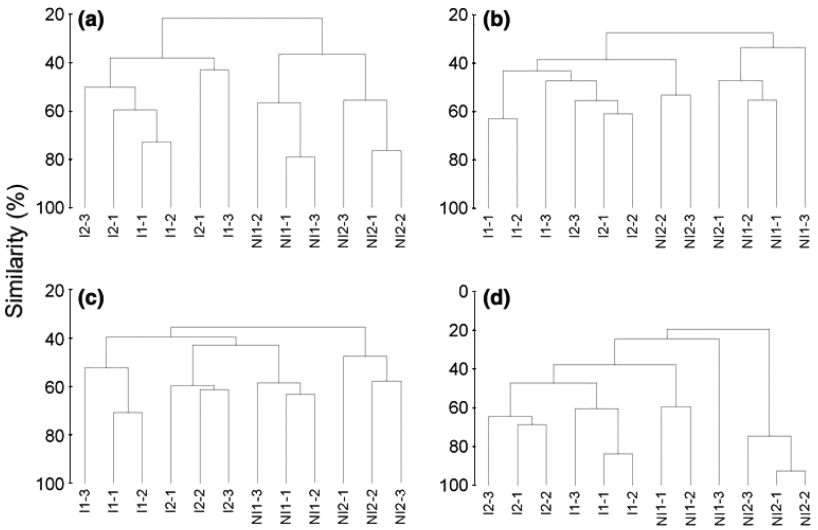

Fig. 2. Cluster analysis of the T-RFLP profiles obtained after digestion of 16S rRNA gene PCR products with the endonuclease MspI. (a) 5 January, (b) 5 August, (c) 6 January, and (d) 6 August.

samples according to copper exposure, and ANOSIM indicated a strong effect of copper in the functional composition of P_{IB}-Type ATPases (Fig. 3), with *R* values for comparison between impacted and nonimpacted groups of 0.776 for 5 January and 1.0 for all the other sampling times. These results clearly indicate that exposure to high levels of copper has distinguishable effects in both the taxonomic and functional composition of bacterial communities. It is also clear from these results that the effect of copper is stronger on the functional composition of those communities, at least for the copper resistance gene analyzed in this work. These observations need additional studies to discard a potential bias because of copA primer pair specificity, that is, the possibility to also detect cadmium-type ATPases (De la Iglesia *et al.*, 2010), overestimating the change on functional profiles. It should be kept in mind, however, that only copper ATPase-related sequences were found by clonal analysis in another study using this primer pair (Pavissich *et al.*, 2010). In any case, the differences observed for grouping according to copper exposure between taxonomic and functional molecular markers can be explained by, at least, two reasons: i) the bacterial communities are able to adapt to copper exposure by changing the variants of resistance genes (i.e. *copA*-like genes) without dramatically altering the community composition, indicating that these genes are either normally present in the community or susceptible to horizontal gene transfer (Coombs & Barkay, 2004; Martinez *et al.*, 2006) or ii) the functional marker is present in a bacterial subgroup where the effect of copper is more clearly observable. The differential incidence of copper on taxonomic and functional markers has been also observed on bacterial communities from copper-exposed sediments (Pavissich *et al.*, 2010).

Short and acute exposure to copper affects the composition of nonimpacted but not that of impacted bacterial communities

To corroborate whether copper exposure was responsible for the observed changes on taxonomic and functional composition of bacterial communities, two different sets of microcosm experiments (*Response Experiments*) were implemented. As well as for natural samples, no significant differences between I and NI treatments were detected for R, H, and E (data not shown). However, the bacterial community T-RFLP profiles showed that both groups of samples react different to copper addition (see Fig. S2). Qualitatively, samples from I-2 site display virtually equal profiles, with no significant changes between treatments (see Fig. S2a and c) and controls (see Fig. S2e). Cluster and ANOSIM of those profiles (data not shown) supported the qualitative assessment and no grouping for I treatments ($R = 0.107$; $P < 0.05$). This indicates that epilithic bacterial communities with a history of long-term copper exposure reached a stable composition that is not altered when facing additional copper exposure. It should be remembered that these communities have been subjected for more than 60 years to selective pressure against copper excess.

In contrast with what was observed for samples from I-2 site, microcosm tests performed with samples from NI-2 site showed clear changes in the community profiles after copper exposure, when compared to the control treatment (see Fig. S2b, d and f). ANOVA of *R*, *H*, and *E* values for the NI treatments showed significant differences between copper treatments and controls ($P < 0.05$). Cluster and ANOSIM of these profiles (data not shown) indicated a strong grouping for NI treatments ($R = 0.944$;

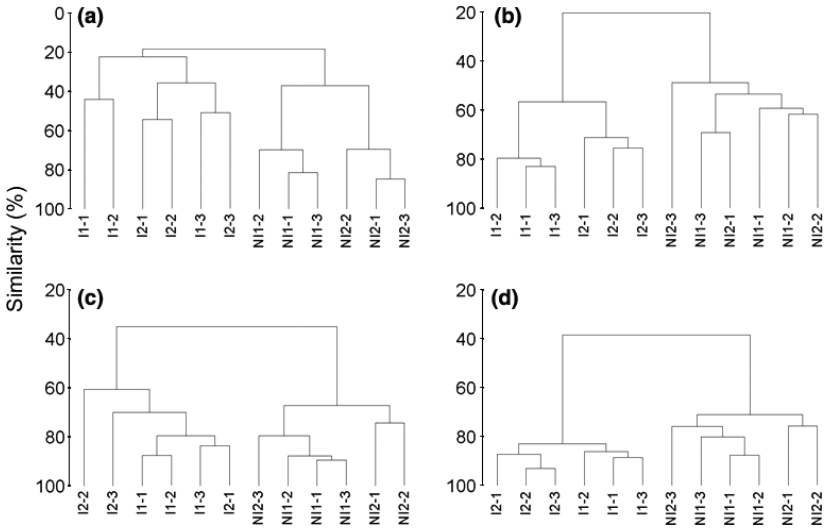

Fig. 3. Cluster analysis of the T-RFLP profiles obtained after digestion of *cop*A gene PCR products with the endonuclease AluI. (a) 5 January, (b) 5 August, (c) 6 January, and (d) 6 August.

$P < 0.05$), with respect to copper addition, indicating that natural, nonexposed bacterial communities quickly respond (48 h) to this metal. In the context of first time effects of copper mining discharges, these results clearly suggest a rapid shift of the bacterial community and that this new 'impacted' community structure persists in time.

A striking observation arose when the effects of copper exposure were compared for NI and I samples. Whereas control treatments (C) had an average similarity of 58%, this value increased to 65% for the 50 μg Cu L^{-1} treatment and to 62% for the 100 μg Cu L^{-1} treatment (compare Fig. S2c-f). This result strongly suggests that copper exposure causes a directional shift of the bacterial community composition, leading to a copper-exposed-like community pattern, independent of the origin of the samples, that is, bacterial community structure from samples exposed for long (I) or short (NI) times to copper excess tends to be similar. In the case of *copA*-based composition profiles, differences were detected between samples, same as observed for natural samples. However, no difference between any of the treatments was detected (data not shown), that is, there is not an increase in similarity of *copA* profiles between I samples and NI samples for any of the treatments. This result suggests that changes in 16S rRNA gene profiles are faster than changes in *copA*-like genes profiles.

Changes in bacterial community structure because of copper exposure are reversed when the metal is removed

To assess whether changes produced by copper can be reverted, a *Reversion Experiment* was set up. The structure of the bacterial communities from controls remained stable and grouped together, showing that copper was more important than the incubation time in altering the taxonomic bacterial community composition (Fig. 4) and that changes produced by copper excess were consistent with what was determined in the *Response Experiment*, and the field samples. The more striking observation came from analysis of changes in the microcosm samples that were exposed for 48 h to copper and then incubated without copper for additional 48 h. In this case, cluster analysis showed that these samples were grouped with the control samples, but not with those exposed to copper (Fig. 4). That was corroborated by ANOSIM, with an *R*-value of 0.49 ($P < 0.05$). This result indicates that the copper effect on bacterial community composition is reverted if the metal is removed and that reversion is as fast as the shift in community composition provoked by the addition of the metal. This behavior may be due to the use of microcosms and does not necessarily represent what may occur in field conditions.

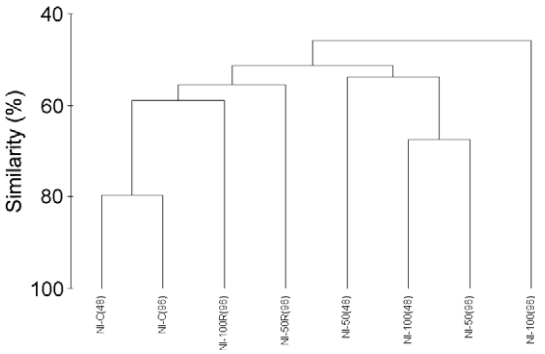

Fig. 4. Cluster analysis of the T-RFLP profiles obtained after digestion of 16S rRNA gene PCR products with the endonuclease MspI, from the '*Reversion Experiment*'. Numbers in parentheses correspond to the incubation time. C indicates the control treatments, and R, the reverted treatments.

The results obtained with the *Reversion Experiment* indicate that epilithic bacterial communities have a high resilience (i.e. the ability of a system to resist damage and recover quickly) to copper excess, a phenomenon that has been observed for other microbial communities in different environments (Girvan *et al.*, 2005). The ability of the bacterial community to recover after a perturbation because of metal excess opens new perspectives about the restoration of environments that have suffered metal contamination. From an ecological point of view, the results obtained with the *Reversion Experiment* are remarkable because bacterial communities support and influence settlement, recruitment, and development of macroscopic organisms (Egan *et al.*, 2001; Matsuo *et al.*, 2003; Wahl, 2008).

The results obtained in this work can be extrapolated to other environments with a history of exposure to high levels of copper (or other metals), as bacterial communities may have evolved common mechanisms to react and adapt to such perturbations. However, additional studies are required before patterns of response to copper exposure can be revealed. The use of both field observations and microcosm experiments along with high-throughput molecular techniques will certainly help to establish the relevance of these adaptation mechanisms.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Representative T-RFLP profiles obtained after digestion of 16S rRNA gene PCR products with the endonuclease MspI.

Fig. S2. Representative T-RFLP profiles obtained after digestion of 16S rRNA gene PCR products with the endonuclease MspI, during the *Response Experiment*.

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